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Original Article

Antioxidant, DNA damage protective, neuroprotective, and α-glucosidase inhibitory activities of a flavonoid glycoside from leaves of *Garcinia gracilis*

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ABSTRACT

The leaves of *Garcinia gracilis* Pierre, Clusiaceae, have been used as flavouring materials in food, with no previous reports of their biological activities and chemical constituents. In this study, the methanolic extract of *G. gracilis* afforded three compounds namely apigenin-8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**), 5-hydroxymethyl-2-furaldehyde, and vanillic acid. All of the isolates were initially evaluated for superoxide anion radical scavenging activity and α-glucosidase inhibitory effects. Compound **1**, which was the major component, showed the most potent activities among these three isolates. Further biological evaluations revealed that compound **1** could prevent the pBR322 plasmid DNA damage induced by the photochemical reaction of riboflavin and protect P19-derived neurons from the oxidative stress condition induced by serum deprivation. It was concluded that the potent biological activities of *G. gracilis* could be attributed to the synergistic effect of compound **1** with other constituents found in the plant.

Introduction

Ageing is an unavoidable phenomenon in living organisms that results in morphological, biochemical, functional and psychological changes in the organism (Moreira et al., 2014). According to a United Nations report, the average life span of the world population has been increasing, and it is estimated that the percentage of elderly people (those aged 60 years or over) will continue to grow and will reach 21.1% by 2050 due to low birth rates and longevity (Rahman, 2007; United Nations, 2013). This increase will result in an increase in age-related chronic diseases, including cardiovascular disease, cancer, diabetes, and neurological disorders, such as Alzheimer's disease and Parkinson's disease. Therefore, it is important to search for anti-ageing medicines, foods, and dietary supplements that are safe and effective to reduce morbidity and provide a good quality of life for elderly individuals (Rahman, 2007).

The excessive production of reactive oxygen species (ROS) and free radicals is considered to be a significant cause of oxidative damage in biomolecules, such as proteins, lipids, and DNA, eventually leading to numerous degenerative diseases. However, these unfavourable effects could be prevented by the consumption of antioxidants to protect the cells from ROS and maintain ROS concentrations at a low level (Gul et al., 2011; Meng et al., 2012). Various medicinal and food plants are rich sources of free radical scavenging molecules, which have strong antioxidant activities (Kuate et al., 2011). In light of these health benefits, the search for antioxidant compounds from natural products has attracted interest.

Moreover, ROS and free radicals are also generated by hyperglycaemia, and may be associated with the metabolic abnormalities that occur in patients with diabetes mellitus (Tiwari et al., 2013). One current approach to the treatment of diabetes and obesity is to control blood glucose levels. α-Glucosidase is a key intestinal enzyme in carbohydrate digestion. The inhibition of this enzyme could delay the carbohydrate hydrolysis process, leading to the prevention of excess glucose absorption in the gut. Acarbose is a well-known α-glucosidase inhibitor that is used to treat type-II diabetes mellitus, but this inhibitors appears to have major side effects, including gastrointestinal disturbance and weight gain (Hollander, 2007). Therefore, it is important to find new

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α-glucosidase inhibitors with fewer side effects, and higher patient approval (Kim et al., 2000; Yin et al., 2014).

The genus *Garcinia* belongs to the family Clusiaceae and includes 390 species that are widely distributed in tropical Asia, Australia, Polynesia, and southern Africa. Twenty-nine species have been reported in Thailand. These species are evergreen trees that range from small to medium in size, and some species can grow up to 30 m in height (Ritthiwigrom et al., 2013; Semwal et al., 2015). Previous studies reported that *Garcinia* plants contain many secondary metabolites and possess various pharmacological effects, including antitumour, antioxidant, anti-inflammatory, and anti-immunosuppressive effects (Seruji et al., 2013).

Garcinia gracilis Pierre, which is also known as Cha-mang or Mak-paem in Thai, is one of the *Garcinia* species that were discovered in Thailand. The ripe fruits and leaves of *G. gracilis* are edible. The leaves of this plant have traditionally been used as flavouring materials in foods (Suksri et al., 2005). The roots are also used as antipyretics folk medicine (Chuakul, 2009). However, no studies have investigated the chemical constituents and pharmacological activities of this plant to date. In this study, our preliminary screening of a methanol extract prepared from the leaves of *G. gracilis* showed a variety of potent biological activities, including superoxide scavenging effects (70.65% inhibition at a concentration of 100 μg/ml), protection against DNA damage (76.46% at a concentration of 100 μg/ml), and neuroprotective effects (100% cell viability at a concentration of 100 ng/ml). This extract also exhibited α-glucosidase inhibitory activity (99.49% at a concentration of 2 mg/ml). These results prompted us to investigate the extract to identify the compounds responsible for these activities.

In the present study, we describe the isolation of compounds **1**–**3** from the leaves of *G. gracilis*, as well as the evaluation of apigenin-8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**), the major isolated compound, for antioxidant, DNA protective, neuroprotective, and α-glucosidase inhibitory activities. Compound **1** was initially identified from the leaves of *Bambusa textilis* (Wang et al., 2012). However, this paper is the first report to describe the presence of a flavonoid glycoside in *G. gracilis*.

Materials and methods

Plant material

Leaves of *Garcinia gracilis* Pierre, Clusiaceae, were collected from Princess Maha Chakri Sirindhorn Herbal Garden in Mueang Rayong District in Rayong, Thailand in February 2011. Authentication was performed by comparison with herbarium specimens in the National Park, Wildlife and Plant Conservation Department of the Ministry of Natural Resources and Environment. A voucher specimen (GG-022554) was deposited in the Department of Pharmacognosy and Pharmaceutical Botany in the Faculty of Pharmaceutical Sciences at Chulalongkorn University in Thailand.

Chemicals

The P19 cell line (ATCC CRL-1857) was obtained from ATCC®, USA. Foetal bovine serum (FBS), new-born calf serum (NCS), alpha minimal essential medium (α-MEM), and an antibiotic-antimycotic solution were purchased from Gibco®, USA. All *trans*-retinoic acid (RA), cytosine-1-β-D-arabinoside, 1:250 porcine trypsin, poly-L-lysine (MW > 300,000), 2,3-bis(2-methoxy-4-nitro-5-sulphonyl)-2*H*-tetrazolium-5-carboxanilide sodium (XTT), α-glucosidase from *Saccharomyces cerevisiae*, *p*-nitrophenyl-α-D-glucopyranoside (pNPG) phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), quercetin, and nitroblue tetrazolium (NBT) were obtained from Sigma–Aldrich®, USA. Riboflavin and acarbose were purchased from Fluka Analytical®, Switzerland. Ethylenediaminetetraacetic acid (EDTA) was procured from May&Baker®, England. pBR322 plasmid DNA was obtained from Vivantis Technologies Sdn. Bhd.®, Malaysia. All solvents were analytical grade and purchased from Merck®, Germany.

Extraction and isolation

Dried and powdered leaves of *G. gracilis* (1 kg) were macerated with MeOH to yield 268.16 g of methanol extract after solvent evaporation. This crude extract was suspended in H_2O and partitioned with EtOAc and BuOH to generate an EtOAc extract (60.23 g), a BuOH extract (119.76 g), and an aqueous extract (42.08 g).

The BuOH extract was then fractionated by column chromatography (CC) (MCI gel, MeOH-H_2O gradient) to generate five fractions (I–V). Fraction II (13.53 g) was separated by CC (silica gel, EtOAc-MeOH gradient) to generate nine fractions (II-A to II-I). Fraction II-E (3.41 g) was further purified on Sephadex LH-20 (MeOH) to generate **1** (781.2 mg). The EtOAc extract was separated by vacuum liquid chromatography VLC (CH_2Cl_2-Acetone gradient) to generate seven fractions (I–VII). Fraction IV (7.58 g) was chromatographed on a silica gel column (CH_2Cl_2-EtOAc gradient) to yield seven fractions (IV-A to IV-G). Fraction IV-C (0.94 g) was further subjected to Sephadex LH-20 (acetone) to generate **2** (20 mg). Fraction V (5.13 g) was then separated by silica gel (CH_2Cl_2-EtOAc gradient) to generate three fractions (V-A to V-C). Fraction V-B (0.57 g) was subsequently purified by Sephadex LH-20 (acetone) to obtain **3** (20.2 mg). All organic solvents used for extraction and isolation were commercial grade and redistilled prior to use. The isolates (**1**–**3**) showed more than 98% purity in the NMR spectrum.

*Apigenin-8-C-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**)*

Yellow amorphous solid; $C_{27}H_{30}O_{14}$; HR-ESI-MS *m/z* 601.1536 $[M+Na]^+$; IR ν_{max}: 3367, 2935, 1654, 1360, 837 cm^{-1}; UV λ_{max}: 215 and 333 nm; ^1H NMR (DMSO-d_6, 300 MHz) and ^{13}C NMR (DMSO-d_6, 75 MHz), as shown in Table 1.

*5-Hydroxymethyl-2-furaldehyde (**2**)*

Brownish oil; $C_6H_6O_3$; HR-ESI-MS *m/z* 149.0216 $[M+Na]^+$; IR ν_{max}: 3390, 1673, 1521 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz): δ 9.59 (1H, s, H-1), 7.38 (1H, *d*, *J* = 3.3 Hz, H-3), 6.58 (1H, *d*, *J* = 3.3 Hz, H-4), 4.65 (2H, s, H-6); ^{13}C NMR (acetone-d_6, 75 MHz): δ 177.2 (C-1), 162.0 (C-5), 152.5 (C-2), 122.9 (C-3), 109.3 (C-4), 56.6 (C-6).

*Vanillic acid (**3**)*

White powder; $C_8H_8O_4$; HR-ESI-MS *m/z* 191.0321 $[M+Na]^+$; IR ν_{max}: 3467, 2917, 2847, 1671, 1433, 1110, 763 cm^{-1}; ^1H NMR (acetone-d_6, 300 MHz): δ 7.62 (1H, *dd*, *J* = 8.4, 1.5 Hz, H-6), 7.57 (1H, *s*, H-2), 6.92 (1H, *d*, *J* = 8.4 Hz, H-5), 3.91 (3H, s, 3-OCH_3); ^{13}C NMR (acetone-d_6, 75 MHz): δ 166.7 (COOH), 151.2 (C-4), 147.2 (C-3), 124.0 (C-6), 122.0 (C-1), 114.6 (C-5), 112.6 (C-2), 55.4 (3-OCH_3).

Determination of antioxidant activity

Assay of DPPH radical scavenging activity

The DPPH radical scavenging assay was performed with a slight modification, as described previously (Likhitwitayawuid et al., 2006). Briefly, the test samples were initially prepared as a solution in MeOH (1000 μg/ml). Each sample was initially evaluated at a concentration of 100 μg/ml, and two-fold serial dilution was

Table 1
^{1}H (300 MHz) and ^{13}C NMR (75 MHz) chemical shifts of compound **1** (DMSO-d_6).

Position	^{1}H (J in Hz)	^{13}C	HMBC (correlation with ^{1}H)
2	–	164.2	2′ and 6′
3	6.78 s	102.6	–
4	–	182.3	–
5	–	160.8	5-OH and 6
6	6.27 s	98.5	5-OH
7	–	162.5	6 and 1″
8	–	104.3	6 and 1″
9	–	156.0	1″
10	–	104.6	3 and 6
1′	–	121.7	3′, 5′ and 3
2′	8.03 d (8.7)	129.1	6′
3′	6.91 d (8.7)	116.1	5′
4′	–	161.4	2′, 3′, 5′ and 6′
5′	6.91 d (8.7)	116.1	3′
6′	8.03 d (8.7)	129.1	2′
5-OH	13.12 s	–	–
Glucose			
1″	4.75 d (9.9)	71.8	2″
2″	4.06 dd (8.7, 9.1)	75.2	1″ and 1‴
3″	3.51 m	80.0	2″ and 4″
4″	3.43 m	70.8	3″ and 6″
5″	3.24 m	81.9	1″
6″	3.75 br d (12.0) 3.53 m	61.3	–
Rhamnose			
1‴	4.97 s	100.5	2″
2‴	3.60 m	70.6	4‴
3‴	3.08 dd (9.0, 2.3)	70.4	1‴ and 4‴
4‴	2.90 t (9.0)	71.6	2‴, 3‴ and 6‴
5‴	2.10 m	68.4	1‴ and 6‴
6‴	0.46 d (6.0)	17.9	4‴

performed for IC_{50} determination. The reaction mixture (200 μl) in each well contained 20 μl of the sample solution and 180 μl of 50 μM DPPH in a 96-well microtiter plate. The reaction mixture was then incubated for 30 min, and the absorbance at 510 nm was measured with a microplate reader. The percentage of DPPH radical scavenging activity was then calculated as follows:

$$\%\,\text{DPPH radical scavenging activity} = \frac{A_c - A_s}{A_c} \times 100,$$

where A_c is the absorbance of the control and A_s is the absorbance of the samples. The experiment was performed in triplicate ($n = 3$), and each experiment consisted of three repetitions. MeOH was used as a negative control. Trolox was used as a positive control and treated under the same conditions as the samples.

Assay of superoxide anion ($O_2^{\bullet -}$) scavenging activity

This assay measures the ability of the test sample to inhibit the reduction of NBT to blue formazan by O_2^- (Chatsumpun et al., 2010). The sample solutions were prepared by dissolving the test sample in a solution of 30% MeOH in potassium phosphate buffer. The reaction (200 μl) was performed by adding 40 μl of sample solution and 20 μl of 750 μM NBT to a mixture of 20 μl of 50 mM potassium phosphate buffer, 100 μl of 266 μM riboflavin, and 20 μl of 1 mM EDTA in a 96-well microtiter plate. The reaction mixture was then illuminated with a fluorescent lamp for 10 min at room temperature. The formation of blue formazan was then monitored based on the increase in the absorbance at 570 nm. A similar reaction mixture was kept in the dark and served as the blank. The percentage of O_2^- radical scavenging activity was then calculated as follows:

$$\%\,O_2^- \text{ radical scavenging activity} = \frac{A_c - A_s}{A_c} \times 100,$$

where A_c is the absorbance of the control and A_s is the absorbance of the samples. The experiment was performed in triplicate ($n = 3$), and each experiment consisted of three repetitions. A solution of 30% MeOH was used as a negative control. Trolox was used as a positive control and treated under the same conditions as the samples.

Assay of DNA protective activity

The inhibitory effect of the test samples on supercoiled DNA breakage was assessed using the agarose gel electrophoresis method (Chatsumpun et al., 2010). Briefly, the test sample was initially evaluated at a concentration of 100 μg/ml, and two-fold serial dilution was performed for IC_{50} determination. Each reaction mixture (10 μl) contained 2 μl of the sample solution, 1 μl of 50 mM potassium phosphate buffer, 5 μl of 266 μM riboflavin, 1 μl of 1 mM EDTA, and 1 μl of 100 ng/μl pBR322 plasmid DNA. The mixture was then illuminated with a fluorescent lamp for 30 min. The same experiment was kept in the dark as a blank. The mixture was subsequently treated with 2 μl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, and 40% sucrose in water), and load onto 0.7% agarose gel. Electrophoresis was conducted at 100 V in a Tris–acetic acid–EDTA buffer. Then, the gel was stained with ethidium bromide (0.5 μg/ml in deionized water) and visualized under ultraviolet light. Images were obtained using a MiniBIS Gel Documentation system and analysed with Gel Quant Analysis (DNR BioImaging Systems, Jerusalem, Israel). The experiment was performed in triplicate ($n = 3$), and each experiment consisted of three repetitions. A solution of 30% MeOH was used as a negative control. Trolox and quercetin were used as positive controls and treated under the same conditions as the samples.

Assay of neuroprotective effects on cultured P19-derived neurons

Cell culture

P19 cells were cultured in P19GM (α-MEM supplemented with 7.5% NCS, 2.5% FBS, and 1% antibiotics-antimycotic solution) in a humidified 5% CO_2 atmosphere at 37 °C. Cells in monolayer cultures were maintained in the exponential growth phase by subculturing every 2 days (Jones-Villeneuve et al., 1982).

Differentiation of P19 cells into P19-derived neurons

Exponentially grown cultures were trypsinised and separated into individual cells. The differentiation of P19 cells was performed by seeding 2×10^6 cells/ml onto a 100-mm bacteriological culture dish containing 10 ml of P19IM (α-MEM supplemented with 5% FBS and 1% antibiotics-antimycotic solution) and 0.5 μM RA. A bulky aggregate of cells was formed in suspension. The cell clusters were then dissociated after 4 days of RA treatment using a 5-ml glass measuring pipette and resuspended on poly-L-lysine-pre-coated multi-well plates (plates coated with 50 μg/ml of poly-L-lysine dissolved in a phosphate-buffered saline (PBS) solution overnight and sterilized under UV light for 30 min) at a concentration of 7×10^4 cells/ml (150 μl/well) in P19SM (α-MEM supplemented with 10% FBS, and 1% antibiotic-antimycotic solution). The cells were then incubated for an additional day. The proliferation of non-neuronal cells was inhibited by the addition of cytosine-1-β-D-arabinoside or Ara-C (10 μM) on the first day after plating, and the medium was renewed every few days. The differentiated P19-derived neurons were used after day 14 of the differentiation process (Jones-Villeneuve et al., 1982; Jones-Villeneuve et al., 1983; MacPherson and McBurney, 1995; Tadtong et al., 2012).

Neuronal viability assay

This method was performed using P19-derived neurons cultured in a 96-well plate. The P19SM supplemented with 10 μM Ara-C was removed after 14 days of the differentiation process, and the sample solutions in DMSO diluted with P19SM containing 10 μM Ara-C were added to obtain the concentrations of 1, 10, 100,

1000, and 10,000 ng/ml. DMSO was added to the cultures at a concentration of 0.5% (v/v) as a solvent control. P19SM supplemented with 10 μM Ara-C was added to the control wells. The cells were kept at 37 °C for 18 h. Then, 50 μl of an XTT solution (1 mg/ml XTT in 60 °C α-MEM with 25 μM PMS) was added, and 150 μl of the medium was taken out. After 4 h of incubation, 100 μl of PBS, pH 7.4 was added to each well. The optical density (OD) was measured at 450 nm using microplate reader. The experiment was performed in triplicate ($n = 3$), and each experiment consisted of three repetitions, with medium at 100% cell viability as a control (Tadtong et al., 2012; Tadtong et al., 2007). The concentration that promoted better survival of the cultured neurons than the control was further evaluated for neuroprotective activity.

Neuritogenicity assay

The assay was conducted with P19-derived neurons cultured in a 96-well plate using the serum deprivation method (Iacovitti et al., 1997; López-Maderuelo et al., 2001; Tadtong et al., 2013). The P19SM supplemented with 10 μM Ara-C was removed after 14 days of the differentiation process, and the sample solutions in DMSO diluted with P19SM containing10 μM Ara-C, the α-MEM supplemented with 10 μM Ara-C, and the 1% antibiotic-antimycotic solution without FBS were added to generate a final sample concentration that enhanced the survival of cultured neurons more than the control. DMSO was added to the cultures at a concentration of 0.5% as a solvent control, followed by P19SM with 10 μM Ara-C in the control wells. α-MEM supplemented with 10 μM Ara-C and 1% antibiotic-antimycotic solution without FBS were used to generate the oxidative stress condition. The cells were kept at 37 °C for 18 h. Cell viability was assayed using the XTT reduction method. The experiment was performed in triplicate ($n = 3$), and each experiment consisted of 3 repetitions, with medium at 100% cell viability as a control.

Assay of α-glucosidase inhibitory activity

The α-glucosidase inhibitory effect was evaluated as described previously, with slight modifications (Sun et al., 2014). This assay measures the enzyme activity by investigating the release of *p*-nitrophenol from the pNPG substrate. Each sample was initially evaluated at a concentration of 2 mg/ml, and two-fold serial dilution was performed for IC_{50} determination. The reaction mixture in a 96-well microtiter plate initially contained 10 μl of test sample and 40 μl of 0.1 U/ml α-glucosidase and was pre-incubated at 37 °C for 10 min. Then, 50 μl of 2 mM pNPG were added to the mixture and further incubated at 37 °C for 20 min. The reaction was then terminated by the addition of 100 μl of a 1 M Na_2CO_3 solution. The amount of *p*-nitrophenol released was measured using a microplate reader to determine the absorbance at 405 nm. The percentage of α-glucosidase inhibitory activity was then calculated as follows:

$$\%\,\alpha\text{-glucosidase inhibitory activity} = \frac{A_c - A_s}{A_c} \times 100,$$

where A_c is the absorbance of the control and A_s is the absorbance of the samples. The experiment was performed in triplicate and each experiment consisted of three repetitions. Five percent DMSO was used as a negative control. Acarbose was used as a positive control and treated under the same conditions as the samples.

Statistical analysis

All analyses were carried out in triplicate ($n = 3$). The data were presented as the mean ± standard deviation (SD). One-way analysis of variance (ANOVA) with the least significant difference (LSD) test was carried out to identify significant differences between the control and experimental groups using SPSS version 18.0 (SPSS Inc., Chicago, IL). Differences were considered significant when $p < 0.05$.

Results and discussion

Isolation and identification of isolated compounds

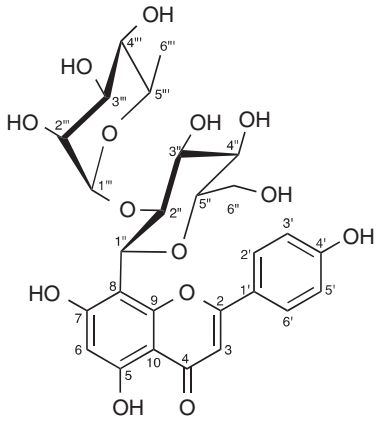
The phytochemical investigation of the MeOH extract from *G. gracilis* leaves yielded a glycosidic flavone compound (**1**) as the major constituent, along with 5-hydroxymethyl-2-furaldehyde (**2**) and vanillic acid (**3**). The ^1H and ^{13}C NMR assignments of compound **1** that were report in a previous study were based on 1D NMR experiments (Wang et al., 2012). The previously reported NMR data are different from our results, especially with respect to the ^{13}C NMR data for the sugar moieties. In this study, the structure of compound **1** was identified based on 1D NMR (^1H, ^{13}C NMR), 2D NMR (^1H–^1H COSY, HSQC, and HMBC), HR-ESI-MS, FTIR, and UV spectroscopy. Compound **1** was obtained as a yellow amorphous solid. The HR-ESI mass spectrum of that compound showed a peak for the $[M+Na]^+$ ion at m/z 601.1536 (calculated for 601.1533), indicating a molecular formula of $C_{27}H_{30}O_{14}$. The IR spectrum of compound **1** exhibited the broad absorption of a hydroxyl group at 3367 cm^{-1} and a carbonyl group at 1654 cm^{-1}. These results, along with UV absorption maxima at 215 and 333, suggested a flavone glycoside structure for compound **1**.

According to the ^1H and ^{13}C NMR spectra of **1** that are listed in Table 1, the ^{13}C NMR data for compound **1** revealed the presence of fifteen aromatic carbon resonances for the aglycone part of the compound and twelve sugar signals, which were detected as one hexose and one deoxyhexose unit. Based on the ^1H NMR data, the apigenin skeleton was identified from the presence of one proton singlet at δ_H 6.78 (H-3) in the aromatic region, as well as two proton signals at δ_H 6.91 and δ_H 8.03 (each 2H, d, $J = 8.7$ Hz) which indicated a *para* substituent at C-4′ of ring B. From the HMBC spectra, the signal at δ_H 8.03 was assigned to two *ortho* protons of ring B (H-2′ and H-6′) based on the correlation of these protons with C-2 of ring C. A hydrogen-bonded hydroxyl proton (s, 5-OH) in ring A was assigned to the sharp singlet peak at δ_H 13.12 in the ^1H NMR spectrum. Based on the HMBC spectra, the proton at 5-OH exhibited correlations with C-6 at δ_C 98.5, C-5 at δ_C 160.8 and C-10 at δ_C 104.6. Consequently, the δ_H 6.27 (1H, s) of H-6 was indicated by its correlation with the carbon signal of C-6 in HSQC.

With respect to the glycosidic part of the compound, two sugar moieties were suggested by the two anomeric protons at δ_H 4.75 (d, $J = 9.9$ Hz, H-1″) and 4.97 (s, H-1‴), and these protons displayed HSQC correlations with the carbons at δ_C 71.8 (C-1″) and 100.5 (C-1‴), respectively. The COSY spectrum was then analysed to establish the proton sequence of the first sugar unit from H-1″ to H-6″, together with the associated multiplicity and coupling constants. A similar experiment was performed for the next unit, allowing us to determine the proton sequence from H-1‴ to H-6‴. Additional ^{13}C NMR data were observed for the first moiety at δ_C 75.2 (C-2″), 80.0 (C-3″), 70.8 (C-4″), 81.9 (C-5″) and 61.3 (C-6″), and for the second moiety at δ_C 70.6 (C-2‴), 70.4 (C-3‴), 71.6 (C-4‴), 68.4 (C-5‴), and 17.9 (C-6‴). These sugar units were identified as α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside based on the HMBC linkage between C-1‴ (δ_C 100.5) of the rhamnose and H-2″ (δ_H 4.06, dd, $J = 8.7$ and 9.1 Hz) of the glucose. From the coupling constant of the anomeric proton (9.9 Hz), the β-orientation of D-glucose was identified. This glycosidic part of the compound was found to be connected to C-8 of the apigenin skeleton based on the correlations of C-8 (δ_C 104.3) with H-1″

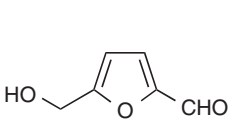
(δ_H 4.75, d, J = 9.9 Hz) and H-6 (δ_H 6.27, s) in the HMBC experiment.

According to the above spectroscopic data, compound **1** was identified as apigenin-8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside.



1

The other known compounds **2** and **3** were identified as 5-hydroxymethyl-2-furaldehyde (**2**) and vanillic acid (**3**) based on a comparison of their NMR spectral data with values available in the literature (Espinoza et al., 2008; Chang et al., 2009).



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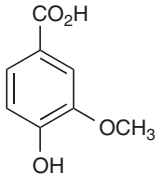

3

Preliminary screening of isolated compounds

Based on a primary screen for superoxide anion scavenging activity at 100 μg/ml, compound **1** and **3** exhibited potent scavenger activity, with values of 96.45% and 82.61%, respectively, whilst compound **2** displayed no activity. The IC_{50} of compound **1** was 23.91 ± 5.37 μM and that of compound **3** was 19.88 ± 1.34 μM. After screening for α-glucosidase inhibitory activity at 2 mg/ml, only compound **1** showed activity, with 96.90% inhibition and an IC_{50} of 0.56 ± 0.01 mM. Compounds **2** and **3** were inactive. Preliminary studies of the biological activities of the isolated compounds demonstrated the antioxidant potential and related effects of compound **1**. Moreover, this flavonoid with an 8-*C* substitution in the A-ring has not been reported previously in *G. gracilis*. Therefore, the presence of an 8-*C* flavonoid glycoside deserved further chemotaxonomic attention and additional investigation of biological activity in this study.

Antioxidant activity

DPPH radical scavenging activity

DPPH is a coloured and stable nitrogen free radical. This assay determines the reducing capacity of an antioxidant by measuring the change of colour from violet to yellow based on its absorbance. Antioxidants can eliminate this free radical via the process of hydrogen atom transfer or electron donation (Mohammed et al., 2015).

Based on the results, the highest scavenging activity was observed for Trolox (IC_{50} = 7.7 ± 1.74 μM). Compound **1** showed a dose-dependent but weaker scavenging effect in this model (IC_{50} = 117.47 ± 14.14 μM) (Table 2, Fig. 1). The weak activity of the apigenin glycoside was consistent with a previous report by Lu and Foo (2001) and indicated the importance of the 3′,4′-dihydroxy group of the B-ring, which is a key factor for scavenging DPPH (Lu and Foo, 2001; Li et al., 2008; Mohammed et al., 2015). In addition, the substitution of a hydrogen atom at the C-8 position in the flavone A ring by the two sugar moieties also decreased the antioxidant activity due to steric hindrance, as those bulky groups reduced access the centre of the DPPH radical (Prior et al., 2005; Zeng et al., 2013).

Superoxide radical scavenging activity

The superoxide radical is a significant cellular free radical and is associated with an increase in oxidative damage in biomolecules due to the production of more powerful reactive species. In our model, O_2^- was generated non-enzymatically by the photo reaction of riboflavin and assayed based on the reduction of NBT to generate blue formazan. However, this process can be inhibited when O_2^- scavengers are present (Chatsumpun et al., 2010).

The O_2^- scavenging activity of compound **1** is shown in Table 2 and Fig. 1. Compound **1** was found to exhibit stronger scavenging activity than Trolox, with IC_{50} values of 23.91 ± 5.37 and 95.66 ± 9.83 μM, respectively. Many studies reported variable results for the O_2^- scavenging activity of flavonoid glycosides depending on the position of glycosylation, the attached hydroxyl group, and the type and number of sugars in the structures (Yokozawa et al., 1997; Zeng et al., 2013; Xiao et al., 2014; Materska, 2015). Based on the studies of Lu and Foo (2001), the catechol and pyrogallol in the B ring are responsible for strong antioxidant activity, but the scavenging activities of the flavone glycosides were all higher than that of Trolox. Those results were consistent with the finding of Yokozawa et al. (1997) that apigenin can inhibit ROS species, even in the absence of 6- or 3′-OH. Those authors also indicated that the linked rhamnose sugar yielded better properties than glucose in their aglycone. Therefore, the high potency of compound **1** in the scavenging of superoxide radicals might also be related to the linked sugar.

DNA protective activity

Antioxidants were found to play a crucial role in protecting against DNA damage caused by ROS species (George et al., 2015). The approach used in this study was based on DNA breakage induced by the photochemical reaction of riboflavin. In the initial stage, the double-stranded pBR322 plasmid DNA, which had a supercoiled conformation (SC) and high electrophoretic mobility, was exposed to ROS species from photosensitized riboflavin. When the DNA was bound by radicals, the DNA strand was broken, resulting in an open-circle conformation (OC) with low electrophoretic mobility. The two forms could be separated by agarose gel electrophoresis (Chatsumpun et al., 2010). The electrophoresis results presented in Fig. 2 revealed the inhibitory activity of compound **1** (Fig. 2A), as well as the positive controls, Trolox (Fig. 2B) and quercetin (Fig. 2C) on DNA cleavage. The DNA products were intact in lanes 1 and 2, but circular supercoiled DNA was found to completely disappear in the light conditions of the control sample. Based on the DNA in lanes 4–7, treatment with all of the samples was found to protect plasmid DNA against the photoreaction of riboflavin as indicated by an increase in the SC form. Fig. 2D shows the concentration-activity relationships of compound **1**. From Table 2, it was observed that both compound **1** (IC_{50} = 23.40 ± 3.37 μM) and quercetin (IC_{50} = 21.01 ± 1.24 μM) were far superior in efficacy to Trolox (IC_{50} = 125.75 ± 29.91 μM). Consistent with a previous $O_2^{\bullet -}$ scavenging assay, the ability of

Table 2
IC_{50} values (μM) of compound **1** isolated from *Garcinia gracilis* for DPPH radical scavenging, superoxide anion radical scavenging, DNA protective, and α-glucosidase inhibitory activities.

Sample	DPPH[a] (μM)	Superoxide anion[a] (μM)	DNA protective[a] (μM)	α-Glucosidase[a] (mM)
Compound **1**	117.47 ± 14.14a	23.91 ± 0.23a	23.40 ± 3.37a	0.56 ± 0.01a
Trolox	7.7 ± 1.47b	95.66 ± 9.83b	125.75 ± 29.91b	–
Quercetin	–	–	21.01 ± 1.24a	–
Acarbose	–	–	–	0.90 ± 0.06b

Dissimilar letters in the same column indicate significantly different values for each parameter at $p < 0.05$ using one-way analysis of variance (ANOVA) with the least significant difference (LSD) test.

[a] The data values are expressed as the mean ± SD of triplicate experiments ($n = 3$), and each experiment consists of 3 repetitions.

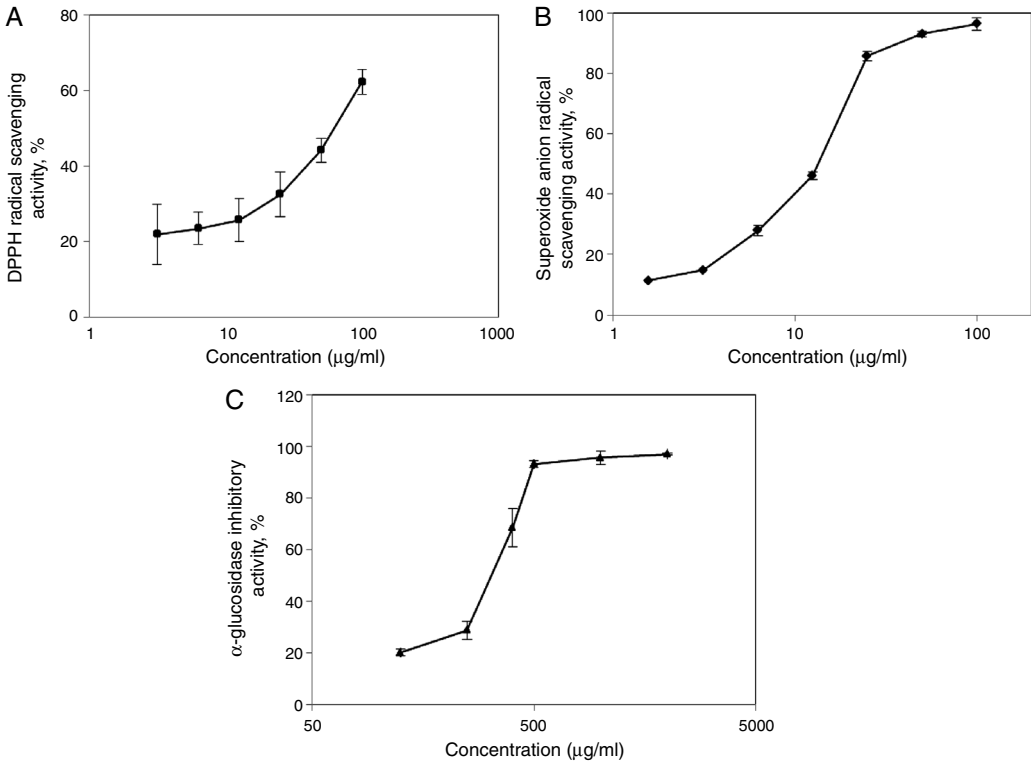

Fig. 1. Concentration-dependent inhibitory effects of compound **1** on: (A) the DPPH radical, (B) the superoxide anion (O_2^-), and (C) the α-glucosidase enzyme. The data are expressed as the mean ± SD of triplicate experiments ($n = 3$), and each experiment consists of three repetitions.

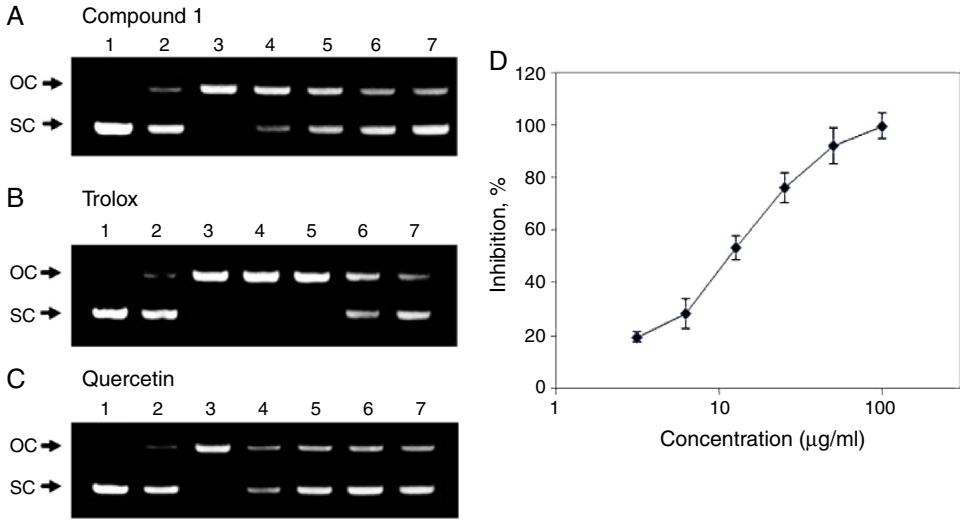

Fig. 2. Protective effect of samples against pBR322 plasmid DNA damage induced by the photochemical reaction of riboflavin. Lane 1 = DNA without treatment; Lane 2 = DNA with riboflavin in the dark condition; Lane 4–7 = DNA with riboflavin in the light condition in the presence of the following samples: (A) Compound **1**, (B) Trolox, and (C) Quercetin at 12.5, 25, 50, and 100 μg/ml. (D) Concentration-dependent inhibitory effects of compound **1** against DNA damage caused by photosensitized riboflavin. The data are expressed as the mean ± SD of triplicate experiments ($n = 3$), and each experiment consists of 3 repetitions.

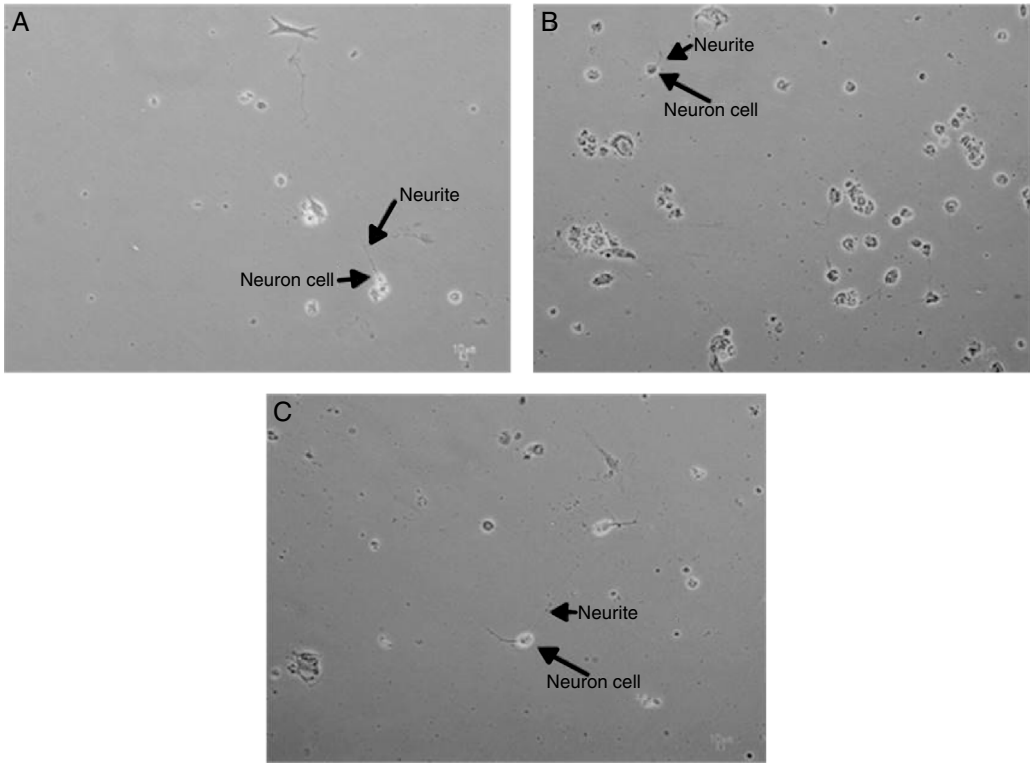

Fig. 3. Phase-contrast micrographs of the neuritogenicity of P-19-derived neurons after 18 h of incubation in: (A) P19SM (α-MEM + 10%, v/v FBS + 10 μM Ara-C) without treatment (control), (B) serum deprivation conditions (α-MEM + 10 μM Ara-C) without treatment (toxic conditions), and (C) serum deprivation conditions (α-MEM + 10 μM Ara-C) treated with compound **1** at 100 ng/ml; scale bar = 10 μm.

flavonoids to protect against DNA damage may be associated with free radical scavenging activity (Boyle et al., 2000).

Neuroprotective activity

Oxidative damage caused by ROS species also occurs in the brain due its large oxygen consumption, its large quantity of fatty acids, and its low level of antioxidant enzymes. Furthermore, neurons cannot promptly recover via mitosis and cell division after they are damaged due to their postmitotic status (Tangsaengvit et al., 2013). P-19 cells, which are a well-known *in vitro* model derived from murine embryonal carcinoma, were differentiated into neurons using retinoic acid. The P-19-derived neurons were found to be irreversibly postmitotic and to contain particular neurotransmitters, such as γ-aminobutyric acid and acetylcholine, which are similar to those found in mature CNS neurons (Tadtong et al., 2012).

The viability of P-19-derived neurons in the presence of compound **1** was investigated using the XTT assay. Compound **1** showed 100% neuron viability at a nontoxic concentration (100 ng/ml). Accordingly, the neuroprotective ability of compound **1** at 100 ng/ml was then evaluated in a serum deprivation model. Serum is a mixture that contains a large amount of proteins and some vital growth factors that are required for the proliferation of cells in culture. The lack of serum induced oxidative stress conditions for the cells, eventually resulting in cell apoptosis (Tangsaengvit et al., 2013). Interestingly, at a concentration of 100 ng/ml, compound **1** significantly protected the cultured neurons against ROS toxicity during serum deprivation-induced oxidative stress, as shown in Fig. 3. Treatment with compound **1** increased the neurite outgrowth of the cultured neurons by approximately four-fold in comparison to the untreated condition (Table 3). Many reports revealed that at a low concentration, flavonoids act as neuroprotective substances via the activation of the mitogen-activated protein kinase (MAP kinase) pathway. In contrast, at high concentrations, flavonoids activate the caspase pathway, leading to apoptosis (Mandel and Youdim, 2004; Tadtong et al., 2013; Williams et al., 2004).

Table 3
Neuroprotective activity during serum deprivation.

Sample	Neuronal viability[a] (%)
Compound 1 (100 ng/ml)	65.74 ± 9.41[b]
α-MEM + 0.5%DMSO	16.34 ± 7.73
α-MEM	16.59 ± 8.13
P19SM[c] + 0.5%DMSO	100.30 ± 0.52
P19SM[c]	100.00 ± 0.00

[a] The data are expressed as the mean ± SD of triplicate experiments ($n = 3$), and each experiment consists of 3 repetitions.

[b] $p < 0.05$ when compared with the toxic condition (α-MEM) and the solvent control of the toxic condition (α-MEM + 0.5% DMSO) using one-way analysis of variance (ANOVA) with the least significant difference (LSD) test.

[c] P19SM is composed of α-MEM + 10% (v/v) FBS.

α-Glucosidase inhibitory activity

α-Glucosidase is a significant enzyme that catalyses the last step of carbohydrate digestion. The inhibition of this enzyme can prevent excessive glucose absorption in the small intestine, thereby controlling blood glucose levels during diabetes therapy without hyperinsulinemia and weight gain (Kim et al., 2000; Xiao et al., 2013). Because a number of *Garcinia* species have been used in the treatment of diabetes and have been proved to exert antidiabetic effects (Ayepola et al., 2014; Baliga et al., 2011; Ryu et al., 2011; Semwal et al., 2015), the isolated compound **1** from *G. gracilis* leaves was investigated for α-glucosidase inhibitory activity. According to the results, compound **1** showed stronger dose-dependent inhibitory activity than the potent drug acarbose with IC_{50} values of 0.56 ± 0.01 mM and 0.90 ± 0.06 mM, respectively (Table 2, Fig. 1).

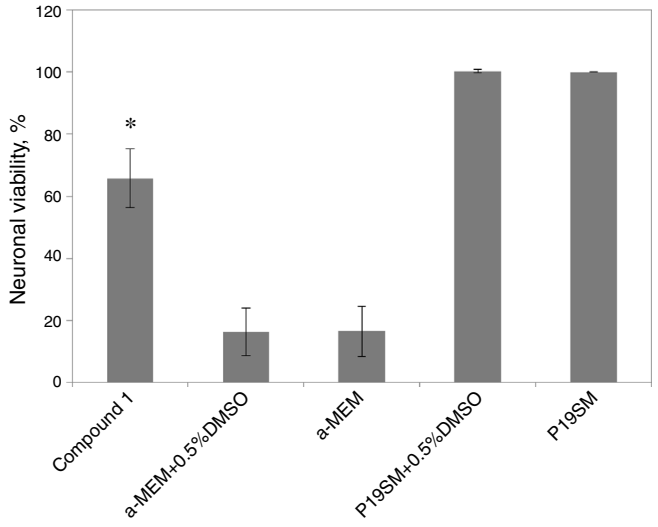

Fig. 4. The effect of compound **1** on the neuronal viability of P-19-derived neurons in the serum deprivation model. The histogram shows the percentage of cell viability relative to vehicle-treated control cultures. Each bar expresses the mean ± SD of triplicate experiments ($n = 3$), and each experiment consists of 3 repetitions. Significant differences were found for the comparisons of compound **1** with the toxic condition (α-MEM) and the solvent control of the toxic condition (α-MEM + 0.5%DMSO) using one-way analysis of variance (ANOVA) with the least significant difference (LSD) test. $^{*}p < 0.05$.

This study is the first report that flavone glycosides from *G. gracilis* exhibit α-glucosidase inhibitory activity, suggesting that this plant could be a potential source of α-glucosidase inhibitors for the treatment of diabetes (Fig. 4).

In conclusion, chromatographic separation of the methanolic extract from the leaves of *G. gracilis* led to the isolation and identification of three compounds, apigenin-8-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside (**1**), 5-hydroxymethyl-2-furaldehyde (**2**), and vanillic acid (**3**). Among these isolates, compound **1** was obtained in the largest quantity and exhibited potential superoxide anion radical scavenging activity, a protective effect against pBR322 plasmid DNA damage, a protective effect against P19-derived serum deprivation, and α-glucosidase inhibitory activity.

Authors' contributions

CS (PhD student) contributed to the isolation and purification of the compounds, the running of the laboratory work, the analysis of the data, and the drafting of the paper. WN contributed to isolation and purification of the compounds. ST contributed to the cell-based assay of neuroprotective activity. PT and KL contributed to the critical reading of the manuscript. BS contributed to the plant collection, the supervision of the laboratory work and critical reading of the manuscript. All authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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